A QUALITATIVE ANALYSIS OF THE EGG-SECRETIONS AND EXTRACTS OF ARBACIA AND ASTERIAS.

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INTRODUCTION.

Although it must have been seen earlier, F. R. Lillie ('12) was the first, so far as I know, to throw emphasis on the observation that when unfertilized *Arbacia* eggs are allowed to stand in a small quantity of sea water, the supernatant liquid takes on a reddish-brown color. The amount of discoloration varies directly as the number of eggs present, the amount of sea water used, and within limits, the time of exposure. If the eggs are now precipitated with the centrifuge, and the fluid above is decanted and filtered through paper, or simply decanted without filtration, it can be very easily shown that substances are present which do not occur in ordinary sea water.

I. ANALYSIS BY MEANS OF SPERM-SUSPENSIONS.

The best method of analysis is that discovered by F. R. Lillie, and although it was not my primary purpose, I have verified a number of his results ('12[1], '13[1], '13[2]). Following Lillie, I shall present my material under four heads: (*A*) Activation; (*B*) Chemotaxis; (*C*) Agglutination; (*D*) Paralysis.

A. Activation.

1. *Iso-Activation.*—As Lillie has pointed out, the phenomena of activation are presented with unequal clearness in different forms. The spermatozoa of *Nereis* for instance are so active in sea water that the effect of the egg-secretion is obscured. With *Arbacia*, the sperm, as is well known, are also active in sea-water, nevertheless a noticeable increase in the rate of their movements can be observed after the addition of a drop of the egg-secretion. *Asterias forbesii* is really much more favorable than either *Nereis* or *Arbacia* for the detection of the activating effect, for here the

spermatozoa are almost motionless. The suspensions I worked with showed only isolated spermatozoa executing occasional spasmodic movements. Upon the addition of the secretion from its corresponding egg, the *Asterias* sperm are thrown into violent activity contrasting sharply with their original state of quiescence.

2. *Hetero-Activation.*—Essentially the same phenomenon described under the head of iso-activation can be observed if *Asterias* sperm are treated with *Arbacia* secretion, and *Arbacia* sperm with that from *Asterias* eggs. As might be expected the phenomenon is more marked with the *Asterias* sperm on account of their original inactivity, although the *Arbacia* sperm are also noticeably accelerated. I have made no experiments capable of deciding whether the activation of the two kinds of spermatozoa is due to the existence in the secretions from the two kinds of eggs of the same substance or of specifically different substances.

3. *Re-Activation.*—Activation is a temporary state, and after certain other reactions have occurred, the spermatozoa are found to be quite immotile. Such spermatozoa, although chemically different ('13[1]) from fresh ones which have never been subjected to the secretions, are nevertheless capable of re-activation. This is shown by the following experiments, in which *Arbacia* sperm-suspensions prepared in each case from a single male were divided into lots, activated by the addition of secretion, observed at intervals, and treated with more secretion as well as fresh eggs. The degrees of activation, reactivation, or movement are given as great, moderate, slight, or zero. In the instances in which eggs were added, fertilizations always took place, but the proportion of eggs that divided varied inversely with the length of exposure of the sperm to the secretion (p. 369).

B. Chemotaxis.

The chemotactic effect of the egg-secretion has been studied very carefully by Lillie, and both methods and results have been described by him at length ('13[1]). I have verified the most essential results on *Arbacia* and have extended them to *Asterias*. The injected-drop method as well as the distribution of sperm about groups of eggs were used as indicators. As Lillie suggests, such results do not make clear the rôle of chemotaxis in normal

ARBACIA SPERM.

Experiment 1.

Treatment.	Lot A, Activation.	Lot A_1, Activation.	Lot A_2, Activation.	Lot A_3, Activation.	Lot A_4, Activation.	Time.
Added secretion	Strong	Strong	Strong	Strong	Strong	11.05 A.M.
	Moderate	Moderate	Moderate	Moderate	Moderate	11.10
Added secretion	.....	Strong	.....	.....	.....	11.10
Added eggs	.....	.....	Strong	.....	.....	11.10
	.....	Slight	Slight	.....	.....	11.11
	Zero	.....	.....	Zero	Zero	3.30 P.M.
Added secretion	.....	.....	.....	Slight	.....	3.30
Added eggs	.....	.....	.....	.....	Slight	3.30

Experiment 2.

Added secretion	Strong	Strong	Strong	.....	.....	12.10 P.M.
	Slight	Slight	Slight	.....	.....	1.10
Added secretion	.....	Moderate	.....	.....	.....	1.10
Added eggs	.....	.....	Moderate	.....	.....	1.10

Experiment 3.

Added secretion	Strong	Strong	Strong	Strong	Strong	12.00 M.
	Slight	Slight	Slight	Slight	Slight	1.00 P.M.
Added secretion	.....	Moderate	.....	.....	.....	1.00
Added eggs	.....	.....	Moderate	.....	.....	1.00
	.....	.....	.....	Zero	Zero	5.00
Added secretion	.....	.....	.....	Slight	.....	5.00
Added eggs	.....	.....	.....	.....	Slight	5.00

fertilization. One of the difficulties is the thigmotactic response of the sperm, as this insures their sticking to surfaces they may chance to meet. It is easy to see how under such circumstances an accumulation about eggs might take place. While not conclusive, experiments in which the surface of the eggs was very materially reduced, and the jelly mass quite obliterated by desiccation, showed that even in this case the sperm collect in great numbers about the eggs. Minute fragments of egg-powder are also centers about which the sperm aggregate in great numbers. I have not observed this when powdered glass or sand were added to the suspensions and as the surfaces in these cases although of different physico-chemical properties, must be assumed to be at least as extensive as in the egg-powder, the marked sperm aggregation about the dried egg-fragments and its absence about the other fragments is certainly in harmony with the idea that chemotaxis is a factor in bringing the sperm to the egg.

C. Agglutination.

1. *Iso-agglutination.*—That egg-secretion is capable of agglutinating sperm has been fully described by Lillie ('12[1], '13[1], '13[2]). In this section I simply wish to add my testimony to his as to the facts in the case—namely that this phenomenon occurs; that it is reversible; that its duration is brief and may be utilized as a measure of concentration; and that sperm once agglutinated, although capable of re-activation, do not agglutinate again.

2. *Hetero-agglutination.*—I have found that the *Arbacia* secretion also agglutinates the sperm of *Asterias*, and that *Asterias* secretion, besides agglutinating its own, has a similar effect on the spermatozoa of *Arbacia*. Thus

1. *Arbacia* sperm + *Arbacia* secretion = agglutination masses described by Lillie.
2. *Arbacia* sperm + *Asterias* secretion = dense, angular masses. Reaction slower than 1.
3. *Asterias* sperm + *Asterias* secretion = masses smaller than 1. Angular.
4. *Asterias* sperm + *Arbacia* secretion = result similar to 3.

At the time when these experiments were performed, the material had practically disappeared, and in consequence the question whether the above hetero-agglutinations are due to the same substances as the iso-agglutinations, or whether each secretion contains both an iso- as well as a hetero-agglutinin, could not be decided. Since hetero-agglutination between *Arbacia* and *Nereis* is not brought about by the same substance ('13[1]) that causes the iso-agglutination, the same relations may hold for *Arbacia* and *Asterias*. It will prove interesting to see whether the agglutinin in *Arbacia* secretion that reacts with the *Nereis* sperm is the same one that agglutinates *Asterias* spermatozoa. If not, it becomes important to discover how many agglutinins are present. In this connection I may refer to an observation already published (13[3]), namely that the *Arbacia* secretion agglutinates the larvæ of *Arenicola*.

3. *Origin of the Agglutinin.*—Lillie has shown that the agglutinin is chiefly located in the outer jelly of the *Arbacia* egg. I have found the same thing to hold true of the *Asterias* ovum. It was also proved by Lillie (13[2]) that if the outer jelly is removed

by shaking, the eggs after two or three washings impart only a weak agglutinating power to the supernatant water. This however increases in the course of time. From this Lillie concludes that the eggs produce the agglutinating substance. Moreover he has shown that they are the only tissue of *Arbacia* that does produce this material.

My own experiments show that the agglutinating substance is located in greatest abundance in the jelly and that the eggs also contain this material. As additional evidence it may be stated that when eggs are inseminated with fairly concentrated sperm-suspensions, the collection and activity of the spermatozoa may be great enough to tear the outer jelly away from the egg. When this occurs, one may suddenly observe great balls of sperm apparently cast off from the eggs and forming huge agglutination masses. In this instance many sperm also remain in contact with the egg, which later shows a typical fertilization membrane, and divides. This point is important in connection with the mistaken idea that the outer jelly is essential for the appearance of the fertilization membrane ('13[4]).

In connection with the existence of agglutinin in the egg, I may refer to experiments with egg-powder in which very effective agglutinations were secured. It cannot well be supposed that every fragment of egg-powder has bits of dried jelly adhering to it.

4. *The Mechanism of Agglutination.*—On this point Lillie's inference based on the *Nereis* sperm, in which because of relatively great size and slow movement, direct observation is possible, can be substantiated by a variety of observations. Lillie noticed that the agglutination is between the heads, and that the tails, at least until the period of paralysis sets in, are not visibly affected. "The adhesion of the heads demonstrates some change in the membrane that renders them sticky" ('13[1], p. 556), and direct observation showed "that in agglutinated masses the heads of many of the spermatozoa are swollen into spherical form and have lost the normal strong refringibility. The change is in this case a very characteristic one indicating a great change in permeability" (*loc. cit.*).

The capacity for influencing the permeability of cells is by

no means limited to the sperm. Thus, as I have pointed out ('13[3]), egg-extract added to blastulæ which have developed in normal sea-water, slows their movements, and increases their volume, indicating a change in permeability. R. S. Lillie has emphasized on numerous occasions the great advantages of *Arenicola* embryos ('13[5] and earlier papers) as indicators of permeability changes which may be registered by the outflow of pigment. When the *Arbacia* secretion is added to the *Arenicola* larvæ, movement is slowed down, the pigment flows freely into the water, indicating an increase in permeability, and a slight and reversible agglutination occurs. Considering all the facts at present available, it seems reasonable to suppose that agglutination is the result of an increase in permeability, and we may imagine that the exudation of material from the cells or the changes that lead to the exudation, render them sticky. Unless some other chemical reaction is involved, it seems to me more likely that the occurrence of agglutination depends upon the exudate. The sperm of *Nereis*, the blastula of *Arbacia*, and the larvæ of *Arenicola* appear to furnish us with three out of four theoretically possible types of cases.

D. Paralysis.

The addition of egg-secretion to a sperm-suspension is followed by activation, a chemotactic effect, and a reversible agglutination. For some time after the agglutination masses have disappeared, the sperm remain quite active, but the rate of their movements decreases until finally they come to a standstill, and appear as though paralyzed. As the re-activations show, this paralysis is not an irreversible state, although the second period of activity never lasts as long as the first, nor is the activity on the whole as great while it lasts. The third activation may be almost momentary. Re-activated sperm are capable of fertilizing the eggs.

II. Qualitative Chemical Analysis of the Egg-Secretion.

Although the observations recorded are important for an understanding of the nature of the egg-secretion, they have been reported at this time and in this connection chiefly for the interest they may have in relation to the factual basis of Lillie's

theory of fertilization ('13[2]). Analyses by other methods were attempted.

It is altogether likely, and in the case of the *Nereis-Arbacia* hetero-agglutination, definitely proved, that the egg-secretion contains more than one substance. The problem therefore presents itself of isolating these bodies. A first step toward orientation has been made by means of certain qualitative chemical tests.

The method of securing egg-secretion finally adopted was suggested to me personally by Dr. F. R. Lillie, and consists in adding to a certain number of "dry" eggs, double their volume of sea-water, and with occasional slight agitation, allowing ten minutes to elapse. At the end of this time the ova were precipitated by 100 revolutions of the centrifuge, and the supernatant fluid, a clear, golden liquid in the case of *Arbacia*, or whitish and opalescent in the case of *Asterias*, was usually carefully decanted without filtration through paper. Such solutions I have adopted as standard. With the *Arbacia* secretion the following tests were made:

1. The solution is gold-yellow in color and clear.
2. The solution is neutral to litmus.
3. Upon cooling to 0° no change was noticeable.
4. Upon boiling the color becomes faintly purple.
5. The purple coloring matter may be removed at least in part if white of egg is allowed to coagulate in the boiling solution.
6. No acid-insoluble precipitate is formed upon the addition of $n/10$ NaOH.
7. 1 or 2 drops conc. HCl produced faint cloudiness which became more distinct on standing.
8. The addition of alcohol produced no visible change.
9. Millon gave a white precipitate with no color change on boiling.
10. The biuret test was negative.
11. HNO_3 gave no ring but a faint cloudiness.
12. The xanthoproteic test gave no precipitate, but the solution turned distinctly yellow.
13. The Adamkiewicz test was negative.
14. Fehling gave no reduction.

15. Bi-subnitrate gave no reduction.

So far as these tests go, and they were repeated several times, it seems likely that reducing substances are absent, and that if proteins are present, their concentration is too low to give the ordinary reactions in their usual form. The opalescence observed upon the addition of acids, and the yellow color gotten in the xanthoproteic test, indicate possibly minute traces of protein but these may come from traces of the egg jelly.

With all the tests that made such experiments possible, the secretion was afterwards tested as to its agglutinating property, and was found in every case to still possess this power, tests 3, 4, and 5. No exact quantitative comparison as to the agglutinating strength before and after boiling was made. Lillie states ('13[1], p. 557) that the agglutinin when boiled and then allowed to stand at 95° for 30 minutes is destroyed in large part, and almost entirely if kept at this temperature for 66 minutes. In some of my experiments the secretion was brought to the boiling point, in others it was boiled 5 minutes. The color change noted always occurred, but the agglutination power appeared undiminished. Both of these results are described in Lillie's paper ('13[1]).

As to the nature of the purple substance, I may say that even if its formation, and presumably with that, the abstraction of something from the original solution makes no measurable difference in the agglutinating power of the secretion, this material may nevertheless be significant in other connections. My reasons for suggesting this are that when a sperm suspension is added to the secretion, traces of this purple color appear; when dilute sperm suspensions are killed by heat the same color is seen; in concentrated suspensions the red dominates over the blue; and in still more concentrated suspensions the color is like that of port wine. This same color also appears in desiccated eggs as well as sperm. From these facts the thought lies near at hand, that we are here dealing with the production of a compound specific for *Arbacia*. Corresponding experiments with *Asterias* do not give this color, nor have I gotten it with oyster sperm. On the other hand, *Asterias* sperm as well as egg-secretion turn a slight salmon-color when boiled. With the exception of these

color differences, the same results in tests, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, and 14, were gotten with the *Asterias* secretion.

III. THE SECRETION OF SUBSTANCES AT FERTILIZATION AND IN HYPERTONIC SEA-WATER.

The unfertilized eggs of *Arbacia* show no noticeable decrease in volume in sea-water even four hours after removal from the ovaries ('14[1]). Fertilized eggs however are measurably smaller in both *Arbacia* and *Asterias* within a few minutes after insemination ('13[3], '14[1]). A change in the same sense seems also to occur in the lamprey ('14[2]).

The decreased volume is probably chiefly due to the loss of water but it can be shown that in *Arbacia* other substances also leave the egg.[1]

The first thing that occurs to one in testing whether at fertilization more soluble substances are secreted than from unfertilized eggs, is to compare the agglutination strength of equal quantities of sea water, with and without sperm, to which equal quantities of unfertilized eggs have been added and allowed to remain for equal lengths of time. Such experiments show that there is more free agglutinin present in the sterile water. The reason for this is not necessarily that the eggs at fertilization secrete less of this substance, for Lillie has shown that the sperm binds the agglutinin, a fact also indicated by the experiments on re-activation without re-agglutination. Moreover it is quite possible that at fertilization other substances are secreted and combine with the agglutinin outside the egg. This alone or in conjunction with the circumstance that a portion of the agglutinin in these experiments would already be bound by the sperm in the infected sea-water would account for the finding of a smaller amount of free agglutinin by any subsequent agglutination test.

[1] McClendon ('09) says of *Arbacia:* "When the egg is fertilized or put in 'membrane-forming' solutions a fluid is extruded which pushes the jelly out from the surface of the egg." As neither the evidence for this statement, nor any reference to the fact that something is also extruded from the unfertilized eggs, are given, I do not know whether my contention is antedated or not. McClendon also states that parthenogenetic reagents, when sufficiently concentrated cause the diffusion of pigment from the eggs into the sea-water. If these concentrations are identical with those most favorable for the initiation of cleavage, there is here an important point of identity between the artificial and normal induction of development.

Such experiments therefore are not capable of deciding whether more or less soluble substance is secreted at fertilization than before. That no agglutinin is secreted after fertilization is complete, has been demonstrated by Lillie's ('13[2]) later work.

The second possibility, that of utilizing the xanthoproteic test suggested itself, for in case the yellow color were not due to traces of dissolved jelly, it might increase as the result of fertilization. As a matter of fact the yellow color gotten from sea-water over eggs inseminated as above is deeper than that given by an equal quantity of sterile water exposed to an equal quantity of unfertilized eggs for the same length of time. This was considered a promising result until it was found that the addition of even a small quantity of sperm increases the density of the color to a marked degree.

A third test however gave comparative results which are not open to this difficulty. In these experiments I made use of the fact that the unfertilized eggs of *Arbacia* discolor the sea-water. Three sets of observations were made. In each of these 1 c.c. of an egg-suspension of given concentration in which the ova were uniformly distributed, was added to each of three tubes, one of which contained 5 c.c. of sterile sea-water, the second 5 c.c. of sea-water infected with just enough spermatozoa to be very faintly opalescent, whereas the third contained 5 c.c. of "double" sea-water, *i. e.*, sea-water which had been boiled down to half its original volume. Color tests were made in the usual way, and the discoloration produced by the unfertilized eggs in a given time was taken as unity. On this basis the following results were obtained in an experiment exactly representative of the others:

	A	*B*	*C*
Time.	5 C.c. Sea-water, 1 C.c. Eggs.	5 C.c. Infected Sea-water, 1 C.c. Eggs.	5 C.c. Double Sea-water, 1 C.c. Eggs.
9.40	Color = 0	Color = 0	Color = 0
9.50	Color = 1	Color = 1.5	Color = 2
9.55	Color = 1	Color = 1.5	Color = 2
9.56	+ 1 c.c. eggs	+ 1 c.c. eggs	+ 1 c.c. eggs
10.10	Color = 2	Color = 3.0	Color = 4

The extra c.c. of eggs was added at 9.56 to see whether the first difference between *A* and *B* could be the result of either the slight

opalescence due to the presence of the small amount of sperm in *B*, or to the small traces of the purple "sperm-agglutinin" compound which was necessarily formed in this tube. However the relative densities of the colors in the three tubes did not change, although the relative effect of the sperm as well as of the purple compound could not have been as great at 10.10 as at 9.50, and 9.55. The same experiment was repeated several times with more dilute suspensions of sperm, and very dense suspensions of eggs. Exactly the same relative discolorations were obtained. I mixed up the tubes on several occasions and asked some one not familiar with the experiment to see if any differences could be noted. I also had the tubes mixed and handed to me for identification. In every case it took but a moment to distinguish the tube in which fertilization had occurred. This was true also in the absence of the tube containing the double sea-water.

One may say therefore that when equal quantities of unfertilized *Arbacia* eggs are allowed to stand for equal lengths of time in equal quantities of sterile and sperm-infected sea-water, the discoloration of the supernatant liquid is greater in the case of the eggs undergoing fertilization. This proves that something in addition to water leaves the eggs at fertilization, a circumstance not at all surprising in view of the fact that, whatever else it may involve, fertilization is accompanied by an increase in permeability.[1] The question whether there is in addition to the quantitative difference, also a qualitative one, must for the present remain open.

[1] Loeb ('13[6]) criticizes McClendon's evidence for increased permeability after fertilization on the ground that more than one interpretation of the experimental evidence is possible. My contention is not that the permeability of fertilized eggs is greater than that of unfertilized, but that there is an increase in the permeability of unfertilized eggs at the moment at which they are being fertilized. This idea is expressed by R. S. Lillie, p. 290, "The Physiology of Cell Division," III. Direct observational support for this view is furnished by F. R. Lillie's work on *Nereis*, and more indirect evidence by my measurements of the rate of secretion by unfertilized eggs as compared with eggs undergoing the process of fertilization. The decreased volume of the *Arbacia* and *Asterias* ovum after fertilization seems to me unintelligible except as the result of an increase in permeability. Why it should be assumed that this increase is more than momentary, I fail to see.

IV. ANALYSIS BY MEANS OF THE RATE OF CLEAVAGE.

A. The Effect of Egg-Secretion in Normal and Alkaline Sea-Water.

A first step toward an answer however has been taken in the form of experiments in which known quantities of egg-secretion were added to normally fertilized eggs and the rate of cleavage compared with controls. The secretions used in these experiments were standard, prepared as before.

Many notes were taken in making these comparisons for it could not be foreseen which details might be utilized as indicators of the relative rates of development. Of all these however only certain data with respect to the 2, 4, and 8-cell stages are reported. This choice depends entirely on the advantage of reporting the more easily verifiable facts, and not because other details are in any way contradictory. Indeed the reverse is true and applies to such stages of the division spindles as can be clearly recognized in the living egg. Similar conclusions also can be drawn from the later stages, although it is much more difficult to tell at a glance whether a certain culture of blastulæ, gastrulæ or plutei, is more or less advanced than a given standard. I wish to emphasize the fact however that these later stages were obtained in large yields in the experimental cultures and were normal, though often somewhat slower of movement.

Several ways of comparing the rates of cleavage are open. Of these the following two were adopted: the time (minimum, in the table) that elapsed between insemination and the first 2, 4, or 8-cell stage seen in a particular culture is given, as well as the interval (maximum, in the table), between insemination and the time at which the cultures were at the height of these respective stages. The results of the second way of counting are in the same sense as the others, but are less accurate since there is greater opportunity for errors of judgment, whereas no error of judgment is likely with respect to the recognition of the first 2, 4, or 8-cell stage seen. The possibility that the particular cases observed were not the first to appear in the cultures of course remains, but a moment's examination of Table I will show that with very considerable errors of this sort consistent results could hardly have been obtained.

The observations were so made that intervals of one minute elapsed between the readings. Thus if *A*, *B*, *C*, *D* represent four culture dishes, *B* was read one minute after *A*, *C*, one minute after *B*, *D* one minute after *C*, and the second reading of *A* was taken four minutes after the first. Under such conditions one could introduce quite a large error from the mere arrangement of the dishes. This was guarded against both by reversing the order of reading from time to time, as well as by changing the order of the dishes in the several experiments. As a matter of fact the significant differences in my tables are always greater than the maximum error which might have resulted from the order of observation.

In the presentation of the results I have not compared the intervals for each of the three stages considered, separately (although the data for such comparison are given), but the average of the intervals that elapsed between insemination and the 2, 4, and 8-cell stages respectively. This procedure further eliminates errors attaching to any specific observation, besides reducing the number of comparisons necessary.

In the right hand division of the following table are contained comparable observations made in sea-water whose C_{OH} had been raised by the addition of 1.75 c.c. *N*/10 NaOH to 100 c.c. sea water. These experiments are included because they serve as checks on those in normal sea water. The theoretical considerations which prompted them were based on the fact brought forward by Loeb ('98) that the development of *Arbacia* is accelerated in alkaline sea-water, and depressed in acid. This is easily verified if normally fertilized eggs are allowed to develop to the blastula stage and are then divided into three lots, one for control, a second to which NaOH is added in the proportion of 1.75 c.c. *N*/10 per 100 c.c. of sea water, whereas the third is acidulated with HCl in the proportion of 1.75 c.c. *N*/10 per 100 c.c. of sea water. In such an experiment gastrulæ, with only here and there a short armed pluteus, predominate in the HCl culture at a given hour; the control at the same time contains a large number of plutei in various stages and some late gastrulæ, whereas the NaOH dish holds practically nothing but complete plutei. Loeb, in the paper referred to attributed this result to

the probable acceleration of the oxidations due to the increase in OH ions. I used the NaOH in conjunction with the egg secretion on the theory that if the secretion affects the oxidations it should antagonize the effect of the NaOH, since the secretion alone, in the concentrations employed, retards the development. As measured by the first three cleavages however, the effects are not antagonistic, but additive, for the NaOH if anything had an effect exactly the reverse of the expected and that actually found for the later stages. This result is in harmony with Loeb's later work. In his recent book ('13[6]), p. 35, he says: "The writer published years ago a paper in which he showed that the development of the eggs of *Arbacia* is retarded and finally inhibited if increasing quantities of acid are added to the sea water. He has since vainly attempted to show that the rate of development of the sea urchin egg can be increased with the increase of the concentration of hydroxylions in the sea water." If the reference here is to the paper cited above, evidence is there given that the increase in hydroyxlions does accelerate the development of *Arbacia*, but this acceleration was not noticed clearly until the day after fertilization. Both results seem really to be correct, only the rate of cleavage is either not accelerated or even depressed, whereas the rate of development from the blastula to the pluteus, is accelerated.

TABLE I.

RATES OF CLEAVAGE IN MINUTES AFTER INSEMINATION.

Cells.	Sea-Water 2 Vols.		Sea-Water 1 Vol. Secretion 1 Vol.		NaOH Sea-Water 2 Vols.		NaOH Sea-Water 1 Vol. Secretion, 1 Vol.		
	Min.	Max.	Min.	Max.	Min.	Max.	Min.	Max.	
2	44′	53′	47′	59′	45′	52′	54′	77′	Experiment I
4	64	74	73	92	52	74	72	93	
8	83	91	.....	.....	74	95	.....	.....	
2	48	52	47	70	53	80	51	70	Experiment II
4	48	71	55	83	80	.....	70	88	
8	71	84	63	88	85	.....	88	.....	
2	39	53	41	66	40	46	41	64	Experiment III
4	42	74	66	91	46	76	.....	77	
8	.....	92	95	.....	82	93	94	.....	
2	45	67	50	74	42	59	61	79	Experiment IV
4	48	72	50	88	44	86	73	109	
8	72	107	98	113	70	98	.....	.....	
Average rate....	55	74	62	82	59	76	76	for first three divisions	
Average rate....	47	65	54	78	50	66	64	82 for first two divisions	

It is apparent from these figures that whether we base our averages on the time that elapses between insemination and the minimum number of a given stage or the maximum, the results are always in the same sense. The special calculations based on the 2 and 4-cell stages have been made for all the columns in order to make fair a comparison of the first three with the last in which the records do not include the 8-cell stage. It seems reasonable to assert that the presence of egg-secretion in certain concentrations retards the early development of the *Arbacia* ovum, and that the presence of NaOH in definite concentrations increases the retardation.

B. The Effect of Egg-Extract in Sea-Water.

Experiments with egg-extract, corresponding to those with egg-secretion were made for the purpose of determining whether the egg contains, and is therefore possibly able to secrete during the brief period of increased permeability incident to fertilization, substances whose effect on the rate of development might be different from that of the secretions heretofore used. Extracts were made by the use of hypertonic sea-water ("double sea-water"), by grinding the eggs with pulverized glass as well as by laking them in distilled water. The quantities and concentrations used corresponded as nearly as possible to the amounts of secretion employed in the earlier experiments although other concentrations were tried. In all cases marked retardation of development was observed. Owing to the fact that the extracts prepared by the three methods are not identical and that their analysis has not been carried far enough as yet, accurate comparisons with the secretion are at this time impossible. However three characteristics of extract-cultures contrasted sharply with cultures developing in the presence of secretion. These were: numerous arrests of development in the early cleavages; much cytolysis; and a very general failure to get beyond the early non-motile blastula. These results indicate a qualitative difference between the secretion and the extract.

V. THEORETICAL.

The heightened rate of oxidation, the increased rate of secretion together with the decrease in volume on impregnation, all suggest

as the essential point in the initiation of development, the relief of antagonistic conditions. When I found that the egg-secretion in certain concentrations actually retards development, I thought I had located the antagonists, and, without knowledge of the earlier and similar suggestion by R. S. Lillie ('09), postulated that initiatory agents are effective "because through increased permeability of the plasma film the egg is enabled to loose substances antagonistic to oxidation" ('13[3], p. 450).

It would of course have been more conservative to say "antagonistic to development," for the retardation brought about by the secretions and extracts may be the outcome of interferences with other conditions and processes, no less essential for normal development, than the oxidations. Indeed Loeb and Wasteneys ('11) have demonstrated the independence of the temperature coefficients of oxidation and cleavage, so that retardation of the latter is not synonymous with a depression of the former. Again the secretion, in the concentrations in which it was employed, brings about abnormal permeability relations which might account for the retarded development, and the possibility that the substances involved have one effect in the concentrations in which they occur in the unfertilized egg, but different effects in the higher concentrations of the experiments, must not be overlooked for good analogies are to be found in the effects of different concentrations of ether (R. S. Lillie, '12[2], p. 373). While it may yet be true that the delaying effect of the secretion is actually the outcome of a depression in the rate of oxidation, proof of this must be sought in further experiments.

However, even if further experimentation should succeed in tracing the retarded development to a decrease in the oxidation rate brought on by the presence of the egg-exudate, a difficulty would still remain, for the surface film of the unfertilized ovum is permeable for the secretion, and so by constant elimination an accumulation of the suspected antagonist to the inhibition point would be automatically prevented. This is the argument applied by Loeb in contraversion of R. S. Lillie's idea that CO_2 might be the antagonist, for inasmuch as CO_2 "is a good agency for calling forth membrane formation" and as only substances capable of diffusing into the egg can have this effect, the egg surface

must be permeable to it before impregnation, and consequently there can be no accumulation that could be relieved as the result of the increase in permeability associated with fertilization.

However two possibilities remain; either the quantitative difference in the rate of secretion between unfertilized ova and ova undergoing fertilization is significant, or there is a qualitative difference as yet undetected between the secretions from the two kinds of eggs. Since "the velocity of segmentation in eggs fertilized by two spermatozoa is identical with that found in eggs fertilized by one. . . . ," we must believe, according to Loeb ('13[6], p. 13) "that the spermatozoan causes development . . . by removing an obstacle to development."

Certain facts and considerations to be dealt with in a later paper, seem to point to the possibility that in addition to the quantitative difference, the secretion at the moment of fertilization differs from the secretion of uninseminated eggs, qualitatively as well. Should further experimentation establish the correctness of this suspicion, then since the rate of oxidation in fertilized eggs is greater than in unfertilized, we could still say, "substances antagonistic to oxidation are eliminated at fertilization" although these substances may be neither CO_2 nor any of the constituents of the secretion from unfertilized eggs. Furthermore their action may be indirect through the inhibition of processes which when set going by their removal allow oxidation to proceed at the heightened rate normal for the fertilized egg. Whatever the final solution of these problems may be, it seems altogether likely that the initiation of development, and with it, the initiation of the processes leading to cell division, are in some way significantly related to the momentary increase in the permeability of the ovum, accompanying the process of fertilization.

VI. SUMMARY.

1. In corroboration of F. R. Lillie, it was found that the egg-secretions of *Arbacia punctulata* exert a chemotactic effect on sperm, and activate, agglutinate, and paralyze them.

2. The egg secretions of *Asterias forbesii* behave in a similar manner toward *Asterias* sperm.

3. *Arbacia* secretion activates, agglutinates, and paralyzes

Asterias sperm, and *Asterias* secretion has the same effects on *Arbacia* sperm.

4. Paralyzed sperm may be reactivated but not reagglutinated.

5. The egg secretions test negatively for reducing substances and do not give the usual protein tests although they were found to be faintly positive to the acid tests and the xanthoproteic. This may be due to traces of the egg jelly.

6. Agglutination may be gotten with dry egg powder.

7. The agglutination reaction very possibly depends on a surface effect.

8. More soluble substances escape from the egg of *Arbacia* in hypertonic sea-water and in sea-water infected with sperm, than from unfertilized eggs in normal sterile sea-water in the same length of time.

9. The egg-secretion in certain concentrations retards development measurably.

10. 1.75 c.c. $N/10$ NaOH added to 100 c.c. of sea water does not accelerate the early cleavages. The retardation noted may well be within the limits of error.

11. NaOH in the same concentration does markedly accelerate the development of blastulæ into plutei.

12. Egg secretion in the concentrations employed + NaOH in the concentration given above, results in a more marked retardation of cleavage than the egg-secretion without the NaOH.

13. Egg-extract, as contrasted with egg-secretion, in addition to retarding development, in a similar manner when employed in a similar manner, results in cytolysis, arrests of development in the early cleavages, and a general failure of the eggs to get beyond the early non-motile blastula.

14. The heightened rate of oxidation in the fertilized egg; the increased rate of secretion in eggs undergoing fertilization; the decreased volume after fertilization all point toward the possibility that initiation of development depends upon the removal of substances, directly or indirectly antagonistic to oxidation. Proof that the egg-secretion in certain concentrations measurably retards development is however insufficient evidence either for the conclusion that it itself is the antagonist, or contains it.

This would not follow even if it were shown that the retardation is the result of depressed oxidation. It is possible however that egg-secretion, at the moment of fertilization differs qualitatively from the earlier secretion, and contains the real antagonists whose inhibitory effect need not necessarily be thought of as having been direct.

15. These suggestions together with the facts upon which they are based, are not necessarily out of harmony with existing prominent theories of fertilization.

UNIVERSITY OF MICHIGAN,
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